APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1997, p. 3104–3110 Vol. 63, No. 8
0099-2240/97/$04.00+0

Evidence for Paralytic Shellfish Poisons in the Freshwater Cyanobacterium *Lyngbya wollei* (Farlow ex Gomont) comb. nov.

W. W. CARMICHAEL,[1] W. R. EVANS,[1] Q. Q. YIN,[1] P. BELL,[2] AND E. MOCZYDLOWSKI[2]

Department of Biological Sciences, Wright State University, Dayton, Ohio 45435,[1] and Departments of Pharmacology and Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, Connecticut 06510[2]

Received 30 January 1997/Accepted 6 June 1997

***Lyngbya wollei* (Farlow ex Gomont) comb. nov., a perennial mat-forming filamentous cyanobacterium prevalent in lakes and reservoirs of the southeastern United States, was found to produce a potent, acutely lethal neurotoxin when tested in the mouse bioassay. Signs of poisoning were similar to those of paralytic shellfish poisoning. As part of the Tennessee Valley Authority master plan for Guntersville Reservoir, the mat-forming filamentous cyanobacterium *L. wollei*, a species that had recently invaded from other areas of the southern United States, was studied to determine if it could produce any of the known cyanotoxins. Of the 91 field samples collected at 10 locations at Guntersville Reservoir, Ala., on the Tennessee River, over a 3-year period, 72.5% were toxic. The minimum 100% lethal doses of the toxic samples ranged from 150 to 1,500 mg kg of lyophilized *L. wollei* cells^{-1}, with the majority of samples being toxic at 500 mg kg^{-1}. Samples bioassayed for paralytic shellfish toxins by the Association of Official Analytical Chemists method exhibited saxitoxin equivalents ranging from 0 to 58 μg g (dry weight)$^{-1}$. Characteristics of the neurotoxic compound(s), such as the lack of adsorption by C_{18} solid-phase extraction columns, the short retention times on C_{18} high-performance liquid chromatography (HPLC) columns, the interaction of the neurotoxins with saxiphilin (a soluble saxitoxin-binding protein), and external blockage of voltage-sensitive sodium channels, led to our discovery that this neurotoxin(s) is related to the saxitoxins, the compounds responsible for paralytic shellfish poisonings. The major saxitoxin compounds thus far identified by comparison of HPLC fluorescence retention times are decarbamoyl gonyautoxins 2 and 3. There was no evidence of paralytic shellfish poison C toxins being produced by *L. wollei*. Fifty field samples were placed in unialgal culture and grown under defined culture conditions. Toxicity and signs of poisoning for these laboratory-grown strains of *L. wollei* were similar to those of the field collection samples.**

The presence in reservoirs and lakes of the southeastern United States of *Lyngbya* sp., a cyanobacterium in the family *Oscillatoreaceae*, has been documented for at least 100 years but has become more common during the past 2 decades. *Lyngbya wollei* (Farlow ex Gomont) comb. nov. was recently identified as the species responsible for these occurrences (28). Benthic mats of this cyanobacterium can reach densities of 6.6 kg (fresh weight) m^{-2} (28) to 13.8 kg (fresh weight) m^{-2} (5). Visible growth of *L. wollei* occurs as dense floating mats, while the majority of the *L. wollei* biomass remains subsurface (28). In the southeastern United States, *L. wollei* overwinters in the form of benthic mats and rises to form surface mats during the warmer months.

Although there have been no reports of toxic compounds being produced by freshwater *Lyngbya* spp., the production of toxic compounds by marine *Lyngbya* spp. is well-known. Lyngbya toxin A and derivatives thereof have been identified as the causative agents for the dermatitis produced by *Lyngbya majuscula* (7). Lyngbya toxin A has also been shown to be a potent tumor promoter and an activator of protein kinase C. Compounds cytotoxic to a murine leukemia cell line (P-388) and an influenza virus (PR8) were found in extracts of a cultured *Lyngbya* sp. Immunosuppressive peptides have also been isolated from *L. majuscula* (16).

Neurotoxic alkaloids have been isolated from species and strains of cyanobacteria in the genera *Anabaena*, *Aphanizomenon*, *Oscillatoria*, and *Trichodesmium* (8). Anatoxin A (antx-A) is a potent postsynaptic, cholinergic nicotinic agonist that causes a depolarizing neuromuscular blockade and has been associated with *Anabaena flos-aquae*, *Anabaena spiroides*, *Anabaena circinalis*, and *Oscillatoria* spp. Anatoxin A(s) [antx-A(s)], which is produced by strains of *Anabaena* (21), is a potent inhibitor of acetylcholinesterase. Saxitoxin (STX) and neosaxitoxin (neoSTX) were shown to be the major neurotoxins present in *Aphanizomenon flos-aquae* (20). In addition, *Anabaena circinalis* water blooms from the Murray River system in Australia have recently been shown to produce paralytic shellfish poisons (PSPs) (4, 15).

When establishing whether compounds that cause acute or acute lethal toxicity exist within a potentially biotoxic cyanobacterium, the general procedure for initial screening is the mouse bioassay. Although there are limitations to the use of this bioassay, it does a good job of allowing one to distinguish between the known neurotoxins and hepatotoxins of cyanobacteria through observation of signs of poisoning and survival times (8). Once toxicity has been established with this bioassay, there are several methods available for cleanup, quantitative analysis, and identification of the toxin(s) (8, 12). The purpose of this study was to determine if the *L. wollei* isolates from Gunterville Reservoir produce any of the biotoxins associated with cyanobacterial blooms and to characterize any biotoxins present.

MATERIALS AND METHODS

Collection of samples. *L. wollei* samples were collected at Guntersville Reservoir, located on the Tennessee River near Guntersville, Ala. Collection of field samples was initiated in November 1991, continued through June 1994, and took place on 10 different days. Sampling locations included Siebold, Ossawintha,

* Corresponding author. Phone: (937) 775-3173. Fax: (937) 775-3320.

Waterfront, Boshart, and County Park. A complete listing and location of sampling locations plus dates can be found in a Tennessee Valley Authority report (9). The samples were stored in an ice chest for transport to the laboratory, cleaned of debris, rinsed with distilled water, lyophilized, and stored at −20°C.

Extraction of samples for chemical and biological assay. The lyophilized *L. wollei* samples were extracted with 25% methanol in water (adjusted to pH 3.5 with acetic acid) or 25% methanol in 50 mM acetic acid at 50 ml per g of material. The mixture was stirred for 1 to 3 h at room temperature and allowed to steep for 12 h at 4°C. The majority of the cellular debris was removed either by filtration through a Buchner funnel with no filter paper or by squeezing the material in the barrel of a disposable syringe. The methanol was removed by evaporation at 25 to 30°C. Depending on the size of the sample, the filtrate was clarified by centrifugation at either 20,000 × *g* for 30 min at 5°C or 16,000 × *g* for 2 min at room temperature.

Bioassay. Estimations of the minimum 100% lethal dose (MLD_{100}) were carried out with duplicate male mice (ICR-Swiss, 16 to 25 g) injected intraperitoneally (i.p.). Symptoms of poisoning and survival times were noted and compared with previous mouse bioassay results from known cyanobacterial toxins. On the basis of survival times (a few minutes to 1 h), it was possible to determine whether neurotoxins or hepatotoxins were present. On the basis of signs of poisoning, it was possible to determine whether antx-A(s) (which results in anticholinesterase signs of poisoning) or antx-A (which results in depolarizing neuromuscular blocking agent signs of poisoning) were present. If these signs were not present, then PSP-type toxins were suspected. When PSP-type toxicity was observed, assays were done according to the Association of Official Analytical Chemists (AOAC) mouse lethality bioassay for PSPs (3). All mouse bioassays were carried out according to the guidelines and protocols of the Wright State University laboratory animal use guidelines. The bioassays were done with extracts of *L. wollei* since adding the lyophilized cells to water resulted in a suspension that could not be drawn into a syringe needle.

Culture of *L. wollei*. The cleaned, rinsed samples were blotted with filter paper, and the mats were cut into ca. 2-cm-long segments. These segments were placed in sterile mesh bags which were immersed in 500 ml of either Z-8 or LM6 medium (9) in beakers and maintained at 26°C at a light intensity of 22 μmol of quanta cm^{-2} s^{-1} with a light/dark cycle of 16 h/8 h. The medium was filtered biweekly through 0.22-μm-pore-size filters. When new filaments grew out of the mesh bags, they were cut into ca. 0.5-cm-long segments and placed at the center of agar (1.0% in Z-8 medium) plates. When uncontaminated trichomes had grown away from these plated filaments, they were cut and transferred to a new agar plate. This process was repeated two to three times, until the filaments were unialgal as indicated by microscopic examination. These filaments were then transferred to 100 ml of liquid medium in 125-ml Erlemeyer flasks and grown until there was sufficient material to use as an inoculum for larger (1-liter) flasks. In some experiments, cells cultured in Z-8 medium were aerated to increase the growth rate. Cells from these flasks were then used to determine the toxicities of the cultures and for isolation of the toxins.

HPLC assay. Techniques for the separation of the three classes of PSPs were carried out on a 5-μm-particle-size 250- by 4.6-mm Inertsil C_8 column by the method of Oshima et al. (25). A Waters 600E pump system was coupled to two postcolumn pumps with a Waters 470 fluorescence detector set at an excitation wavelength of 330 nm and an emission wavelength at 390 nm. Data collection and storage were carried out with a Waters 2010 Millennium Chromatography Manager. Qualitative standards of gonyautoxin (GTX) 1 through GTX4 and a quantitative STX standard were obtained from S. Hall, Office of Seafood, Center for Food Safety and Nutrition, U.S. Food and Drug Administration, Washington, D.C. Quantitative standards of the C toxins, GTX1 to -6 (including decarbamoyl GTX2 and -3), neoSTX, and STX were kindly provided by Y. Oshima, Tohoku University, Sendai, Japan. In some experiments, the GTXs and the STXs were separated in one run by the addition of 10% acetonitrile to the mobile phase used to separate the GTXs. Prior to high-performance liquid chromatography (HPLC), the samples were first centrifuged for 2 min at 16,000 × *g* and 4°C and the supernatant was then either subjected to a C_{18} Sep-Pak solid-phase extraction or centrifuged through a 5,000-molecular-weight-cutoff filter.

SXT binding assay. Tissue preparation was carried out as follows.

(i) Saxiphilin. Adult bullfrogs (*Rana catesbeiana*) were purchased from Connecticut Valley Biological Supply Co., and adult garter snakes (*Thamnophis sirtalis*) were collected in Benton County, Oreg. Animals were handled according to the guidelines of the Yale University Animal Care and Use Committee. Frogs and snakes were anesthetized with tricaine methanesulfonate and sodium brevital, respectively (18). Blood was collected in the presence of heparin, and plasma was prepared by centrifuging whole blood for 30 s at 15,000 × *g*. Bullfrog and snake plasma specimens were stored at −75°C and used as sources of saxiphilin for [^{3}H]STX binding assays as described previously (19).

(ii) Brain microsomes. Whole rat brains (10 g) were homogenized in 80 ml of sucrose buffer (0.3 M sucrose, 10 mM morpholinepropanesulfonic acid [MOPS]-NaOH [pH 7.4], 3 mM NaN_3, 5 mM EDTA) at 4°C in the presence of protease inhibitors (4 μM pepstatin, 4 μM leupeptin, and 200 μM phenylmethylsulfonyl fluoride). The tissue was homogenized for 30 s with a Tissumizer (Tekmar, Cincinnati, Ohio), the homogenate was centrifuged at 2,000 × *g* for 15 min, and the supernatant was saved. The pellet was rehomogenized in 80 ml of sucrose buffer and centrifuged as before. The combined supernatant was centrifuged for 45 min at 100,000 × *g*. The pellet from this combined supernatant was resuspended in sucrose-free MOPS-NaN_3-EDTA buffer and centrifuged for 15 min at 7,700 × *g*. The supernatant was centrifuged for 45 min at 100,000 × *g*, and the final pellet was resuspended in 0.3 M sucrose buffer at a concentration of ~5 mg of protein ml^{-1} and stored in aliquots at −75°C.

TABLE 1. Frequency distribution of toxicity in field samples of *L. wollei* collected from Guntersville Reservoir

MLD_{100}, mg kg^{-1}[a]	No. of samples	% of total samples (n = 91)
150	2	2
250	13	14
500	25	27.5
1,000	18	20
1,500	8	9
NT[b]	25	27.5

[a] Based on the weight of lyophilized samples.
[b] NT, nontoxic at 1,500 mg kg^{-1}.

The standard assay mixture for a [^{3}H]STX binding competition assay with unlabeled STX (Calbiochem-Novabiochem) or *L. wollei* extracts contained 4.2 nM [^{3}H]STX (Amersham), 20 mM MOPS-NaOH (pH 7.4), 0.1 mM EDTA, and either 0.2 M choline chloride (for garter snake or bullfrog serum) or 0.2 M sodium chloride (for rat brain microsomes) in a final volume of 0.35 ml. The binding reaction was initiated by the addition of plasma or microsomes and equilibrated for 1 to 2 h at 0°C before removal of the free toxin at 4°C by passing triplicate 100-μl aliquots of the reaction mixture, followed by a wash solution of 0.5 ml of 100 mM Tris-HCl, pH 7.2, through small cation-exchange columns. The columns contained 1 ml of the microporous resin AG 50W-X2 (Tris+ form, 100/200 mesh; Bio-Rad). Bound [^{3}H]STX was eluted directly into vials and quantitated by liquid scintillation counting. Specific binding is defined as the difference in binding in the absence and in the presence of 5 nM STX. The concentration of total [^{3}H]STX binding sites in the assay was adjusted so that specific binding was ≤20% of the total [^{3}H]STX in the assay. Dilutions of standard STX and the *Lyngbya* extract were prepared in 10 mM citrate buffer, pH 5.2.

Binding competition data were analyzed by plotting the ratio, f, of specifically bound [^{3}H]STX measured in the absence and in the presence of competitor toxin as a function of the total concentration of competitor on a logarithmic axis. Data were fit to the following logistic equation by using the nonlinear fitting utility of the Sigmaplot software (Jandel):

$$f = K_{0.5}{}^n/(K_{0.5}{}^n + [\text{toxin}]_{\text{free}}{}^n) \quad (1)$$

In equation 1, $K_{0.5}$ is the concentration of unlabeled toxin which gives half-displacement of binding (f = 0.5) and n is a pseudo-Hill coefficient. In order to verify the possible presence of Na channel blocking toxins in *L. wollei*, single-channel experiments were done according to the method of Guo et al. (11).

Screening assays. To determine whether neurotoxins (anatoxins) or hepatotoxins (microcystins) were present at low levels, below the mouse bioassay's limit of detection (1 to 2 μg/g of cells), a sensitive immunoassay-enzyme assay was used for microcystin detection and a separate enzyme assay was used for determination of antx-A(s). The immunoassay is based on a polyclonal antibody raised against microcystin LR (10), and the colorimetric protein phosphatase inhibition assay measures the biological activity (i.e., potential toxicity) of microcystins in the sample (2, 30). The presence of antx-A(s) was determined by a colorimetric acetylcholinesterase inhibition assay (21, 22).

RESULTS

The estimated MLD_{100} (i.p.) values of the *L. wollei* samples collected at Guntersville Reservoir ranged from 150 mg kg of lyophilized *L. wollei* cells^{-1} to a nontoxic 1,500 mg kg^{-1}. Of the samples, 72.5% were toxic, with the majority of the samples being toxic at an MLD_{100} of 500 mg kg^{-1} (Table 1). The majority of the *L. wollei* samples that appeared healthy, as judged by the greenish blue-black coloration of the filaments, were toxic, while samples that were nontoxic had been abraided into a heterogeneous, ball-like material and were more brownish yellow in color. The latter type of sample was usually found along shoreline areas. Both benthic and surface mats were toxic. The toxicity signs observed in the mouse bioassay were typical of either antx-A or PSP neurotoxins. Death usually occurred within 20 to 30 min and was preceded by dyspnea, muscle fasciculations, and convulsions. There was

TABLE 2. STX equivalents and mouse units in *L. wollei* samples collected in June 1994

Sample site	STX equivalents[a]	MU[b]
Boshart benthic	15	71
Boshart island	8	36
Boshart 3A	37	156
County Park surface	0	0
Ossawintha surface	24	104
Ossawintha dock	5	22
Siebold surface mat	40	170
Boshart bottom mat	58	248

[a] Micrograms of STX equivalents per gram (dry weight) of lyophilized *L. wollei* cells.

[b] MU, mouse units per gram (dry weight) of lyophilized *L. wollei* cells, as calculated by the AOAC mouse bioassay method (3).

no indication of salivation, which is observed in mouse bioassays when antx-A(s) is present (21). Since the preliminary extraction and chromatography results indicated that antx-A was not present, work focused on determining if PSP is produced by *Lyngbya* cells. With the AOAC mouse bioassay method for determination of PSPs, the amount of STX equivalents in the *L. wollei* samples assayed ranged from 0 to 58 μg g (dry weight)$^{-1}$ (Table 2).

Fifty field samples from healthy filaments collected in the summer to fall were placed in unialgal culture. There was no consistent correlation between the toxicity of the field samples and the toxicity of the cultures obtained from the corresponding field samples. The toxicity of the cultured *L. wollei* cells was either the same, less than, or greater than that of the corresponding field sample (Table 3). For example, Siebold benthic and Ossawintha bottom mat samples showed toxicity as field samples, but isolates cultured from them were not toxic. This could have been due to growth of only nontoxic filaments from a sample having both toxic and nontoxic filaments or to the halting of toxin production when the filaments were cultured. Extraction and characterization of the toxins from these cultures have been carried out and are being reported elsewhere. We were unsuccessful at obtaining axenic cultures, mostly due to the heavy sheath surrounding the filaments. All cultures grown, however, were free of contaminating algae, invertebrates, and fungi. We cannot rule out the possible role of noncyanobacterial microorganisms in toxin production, but all of our observations and tests to date support the production of toxin by the cyanobacterium.

There was no indication of any inhibitory activity in the *L.*

TABLE 3. Comparison of mouse toxicities of field samples with those of *L. wollei* cultured from the corresponding field samples

Sample[a]	MLD_{100} (mg kg^{-1})	
	Field sample	Culture
W-Be	500	500
S-Be	150	NT[b]
SOC #7	500	500
Os-Bm	250	NT
Os-F.I. #30	250	250
Os-S.S.	NT	500
S-Ma #5	NT	250

[a] Abbreviations: W-Be, waterfront benthic; S-Be, Siebold benthic; SOC #7, outside Siebold cage no. 7; Os-Bm, Ossawintha bottom mat; Os-F.I. #30, Ossawintha floating island no. 30; Os-S.S., Ossawintha south side; S-Ma #5, Siebold marina no. 5.

[b] NT, nontoxic at 1,500 mg kg^{-1}.

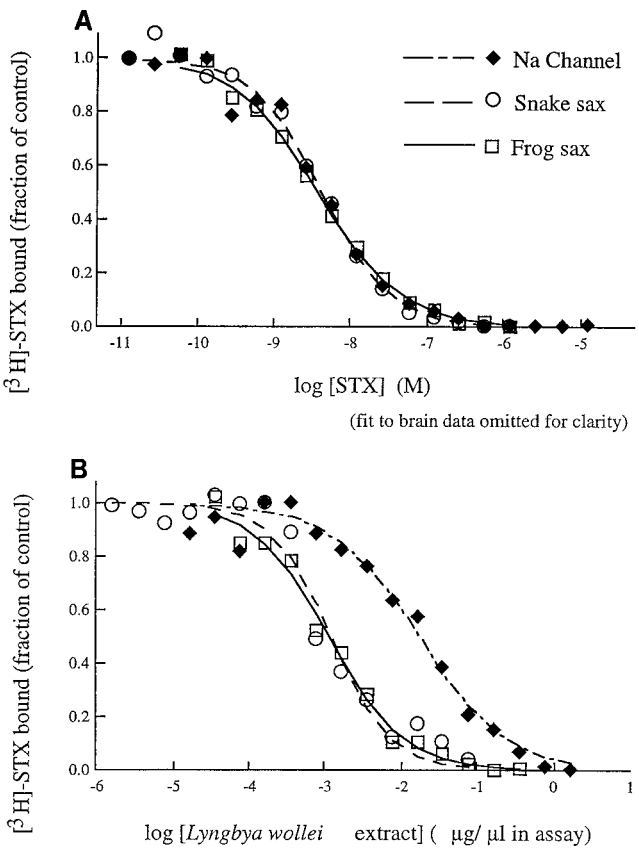


FIG. 1. Inhibition of binding of [^{3}H]STX to Na channels in rat brain synaptosomal membranes, frog saxiphilin in bullfrog plasma, and snake saxiphilin in garter snake plasma. The following parameters were found to fit (solid and dashed lines) the equation $f = K_{0.5}{}^n/(K_{0.5}{}^n + [\text{toxin}]_{\text{free}}{}^n)$. (A) STX displacement: garter snake (Snake sax), $n = 0.96$, $K_{0.5} = 6.13$ nM; bullfrog (Frog sax), $n = 0.81$, $K_{0.5} = 5.59$ nM; rat brain (Na Channel), $n = 0.88$, $K_{0.5} = 6.24$. (B) *Lyngbya* extract-STX competition data fit to $x^n/(x^n + a^n)$: garter snake, $n = 1.11$, $k = 0.0012$ μg/μl; bullfrog, $n = 0.892$, $k = 0.0013$ μg/μl; rat brain, $n = 0.755$, $k = 0.017$ μg/μl.

wollei extracts that were screened for the inhibition of acetylcholine esterase as an indication of the presence of antx-A(s). Enzyme-linked immunosorbent assays (ELISAs) showed that there were no extract components that bound to the microcystin antibody. In addition, the protein phosphatase inhibition assay, which would indicate the presence of microcystins and is a complimentary assay measuring the activity of any microcystins present, was negative.

The purpose of the [^{3}H]STX binding assays was to investigate whether toxic extracts of *L. wollei* contain compounds that could interact with the specific STX binding site that is known to be associated with Na channels (23) or saxiphilin (19). To pursue this question, inhibition of [^{3}H]STX binding was studied with Na channel binding sites in rat brain synaptosomal membranes, frog saxiphilin in bullfrog plasma, and snake saxiphilin in garter snake plasma. Figure 1A summarizes control experiments that established the validity of the assay for each site. The concentration dependence of inhibition by unlabeled STX for each of the three test binding sites is well described by a one-site model of binding competition as noted by pseudo-Hill coefficients of $n \simeq 1.0$ for fits to equation 1 (Fig. 1, legend). The closely overlapping data in Fig. 1A also indicate that the affinities of STX for the three different binding sites are virtually indistinguishable in this assay. This is consis-

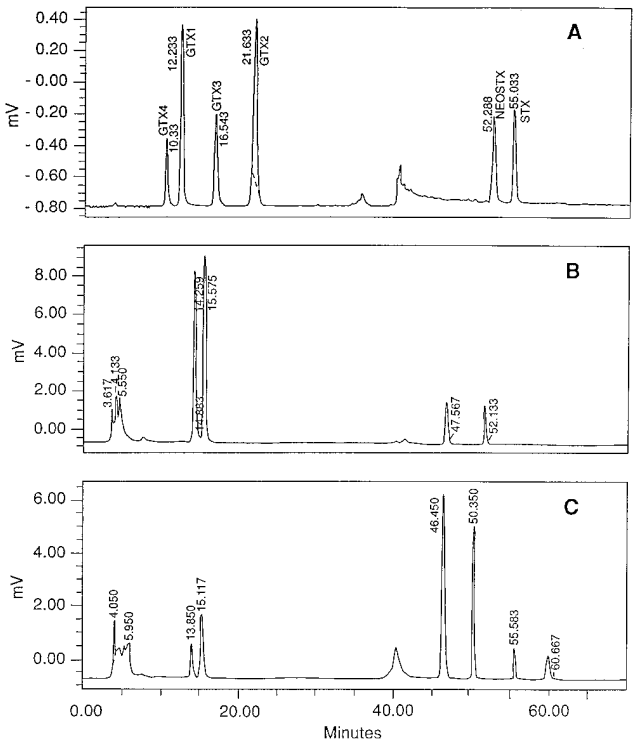


FIG. 2. HPLC chromatograms of PSP standards. (A) GTX1 to -4, neoSTX, and STX; (B) a field sample collected in October 1993; (C) a field sample collected in July 1993. The mobile phase consisted of 10 mM ammonium phosphate–2 mM heptane sulfonate, pH 7.1, for 25 min, at which time 10% acetonitrile was added.

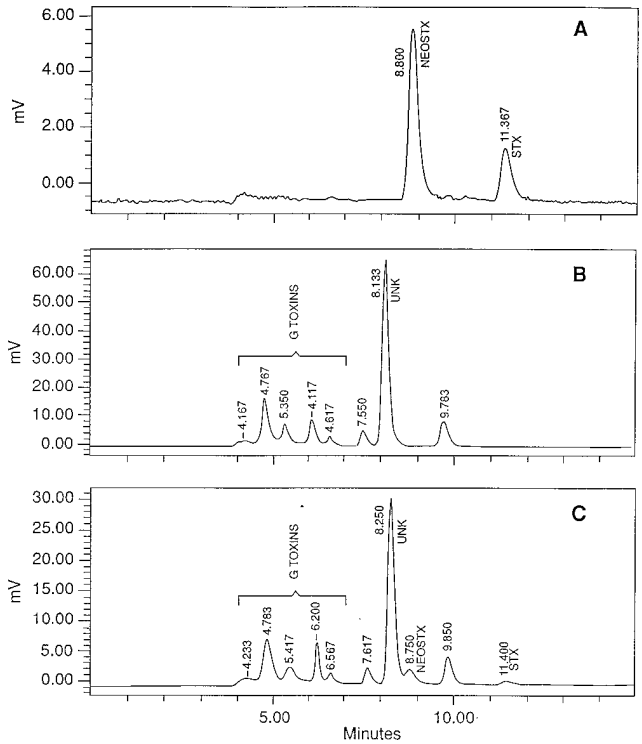


FIG. 3. HPLC chromatograms of STX and neoSTX standards (A), a field sample (B), and a second field sample plus STX standards (C). The mobile phase consisted of 94% 30 mM ammonium phosphate–2 mM heptane sulfonate, pH 7.1, and 6% acetonitrile. UNK, 8.250 and 9.850.

tent with the very similar dissociation constant (K_D) values for [^{3}H]STX that have been measured previously: K_D = 0.22, 0.2, and 0.24 nM for rat brain (23), frog saxiphilin (18), and snake saxiphilin (5a), respectively.

Figure 1B shows the results of similar experiments using an *L. wollei* extract as the competitor liquid instead of STX. We found that the extract readily inhibited binding of [^{3}H]STX to the three types of binding sites. However, the displacement curves obtained for frog and snake saxiphilin are indistinguishable, with the slope of the line (k) $\simeq$0.001 μg/ml, whereas that for rat brain Na channels is shifted to the right by more than 1 log unit (k = 0.017 μg/ml). These results imply that the *L. wollei* extract contained a compound(s) that effectively competed with [^{3}H]STX for binding to each site. However, brain Na channels clearly had a lower affinity for the unknown compound(s) in the extract than for the two saxiphilins. As verification of the presence of Na channel toxins in the *Lyngbya* STX extract, single-channel experiments performed according to the procedure of Guo et al. (11) revealed blocking activity characteristic of low-affinity STX derivatives (data not shown).

Figure 2 shows the HPLC chromatograms of a standard PSP mixture including GTX1 to -4, STX, and neoSTX (Fig. 2A) and of two field samples, one sample of which was collected in October 1993 (Fig. 2B) and the other of which was collected in July 1993 (Fig. 2C). Both field samples had MLD_{100} values of about 250 mg kg^{-1}. These chromatograms indicate that toxins with retention times similar to those of known GTX toxins were predominant in the October sample while two unknown fluorescent compounds that eluted prior to neoSTX were the predominant toxic compounds in the July sample. The HPLC profiles of the majority of the field samples tested were similar to that shown in Fig. 2C.

The HPLC chromatograms of some field samples analyzed for the presence of STX and neoSTX (Fig. 3A) showed the presence of a toxic compound (UNK) (as measured by the mouse bioassay) which eluted just prior to neoSTX and between neoSTX and STX (Fig. 3B). Cochromatography of such a field sample with neoSTX and STX indicated that the compound did not coelute with either neoSTX or STX (Fig. 3C). Since decarbamoyl STX would also elute between neoSTX and STX, this compound may be decarbamoyl STX; it may instead be a new PSP derivative.

Nine toxic field samples were analyzed for the presence of C toxins, but thus far there has been no indication that *L. wollei* contains this type of toxin. Figure 4 shows the HPLC chromatograms of standard C toxins C_1 and C_2 (Fig. 4A) and three representative field samples (Fig. 4B to D).

In other HPLC experiments, extracts of a toxic field sample, collected in July 1993 from the Siebold sample site, were passed through C_{18} solid-phase extraction columns and the eluant was lyophilized. The lyophilized material was suspended in water (50 to 100 mg ml^{-1}) and chromatographed on a C_{18} reversed-phase column. The eluant was monitored by UV absorption (225 nm), and the mouse bioassay was used to check the fractions for toxicity. Two toxic fractions were observed, one eluting at 7 to 9 min and the other eluting at 21 to 23 min. These toxic fractions were then rechromatographed under the conditions used for the decarbamoyl GTX, and retention times were compared with those for decarbamoyl GTX2 and -3. The fraction eluting at 7 to 9 min contained three toxic peaks, two of which had retention times the same as those of decarbamoyl GTX2 and -3 (Fig. 5). The fraction eluting at 21 to 23 min

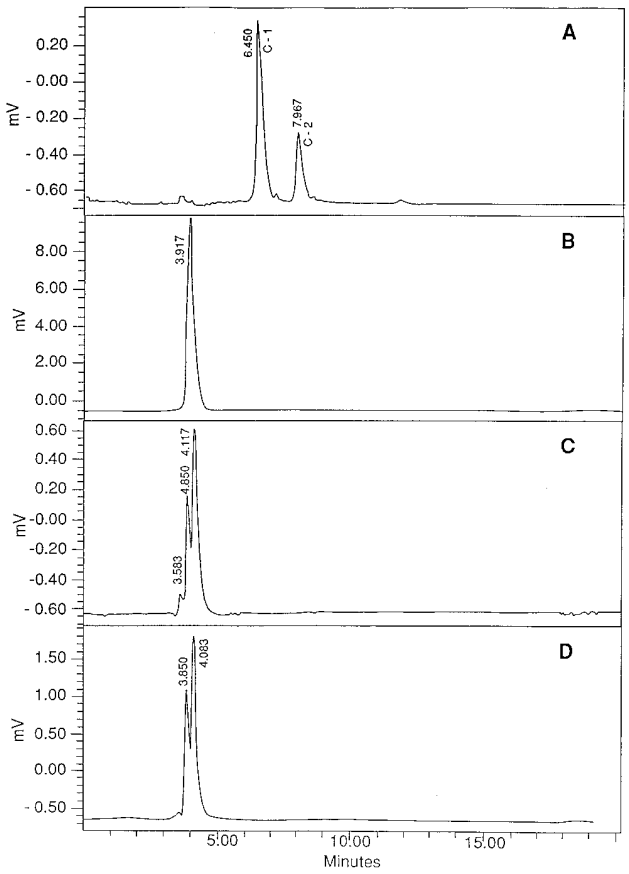


FIG. 4. HPLC chromatograms of the standard C toxins C_1 and C_2 (A) and three toxic field samples (B, C, and D). The mobile phase consisted of 1 mM tetrabutyl ammonium phosphate, pH 6.0.

contained two fluorescent compounds which eluted at ca. 46 and 50 min under the HPLC conditions employed in the study shown in Fig. 2 (data not shown). These later toxic peaks are being analyzed further, with the preliminary structure data indicating that they are related to but different from the decarbamoyl GTXs (24a).

DISCUSSION

To date, two species of cyanobacteria were known to produce PSPs. The biological and chemical data presented in this paper support our conclusion that *L. wollei* is very likely the third cyanobacterial genus to produce PSPs. Our *L. wollei* field samples and laboratory cultures were much less toxic in the mouse bioassay than either *Anabaena circinalis* (4) or *Aphanizomenon flos-aquae* (20). The toxicities of the majority of the field samples (MLD_{100} values, ca. 500 mg kg^{-1}) of *L. wollei* collected at Guntersville Reservoir are approximately seven times less toxic than those of the majority of field samples of the *Anabaena circinalis* blooms in Australia (MLD_{100} values, 75 to 150 mg kg^{-1}). The lowest MLD_{100} of the *L. wollei* field samples was 150 mg kg^{-1}, while that of *Anabaena circinalis* was 30 mg kg^{-1}. The lowest MLD_{100} of the cultured *L. wollei* samples was 250 mg kg^{-1}, while that of *Anabaena circinalis* was 17 mg kg^{-1} (14) and that of *Aphanizomenon flos-aquae* was 5 mg kg^{-1} (20). Mouse bioassays conducted according to the AOAC protocol for PSPs indicated that some of the *L. wollei* samples contained 58 μg equivalents of STX (dry weight) of the cyanobacterium^{-1}. Toxic bivalve samples have been found to have STX equivalents in the range of 900 to 2,000 μg 100 g (wet weight)$^{-1}$. The number of mouse units, 8,000 g^{-1}, found in *Aphanizomenon flos-aquae* (20) was ca. 30-fold more than that found in the most toxic *L. wollei* samples, 258 g^{-1}. All of the mouse toxicity results are consistent with the chromatographic data showing that the more toxic STX and neoSTX PSPs are not present in *L. wollei*. If they had been present, then the number of mouse units would have been closer to that found in the *Aphanizomenon* and *Anabaena* spp. that do produce these toxins. The numbers of mouse units present in the *L. wollei* extracts are, however, consistent with those of the less toxic decarbamoyl GTX2 and -3, whose presence is supported by the HPLC data.

To support the more widespread occurrence of toxic *L. wollei* strains, we found that a sample collected at Lake Blackshear, Ga., by L. Dyck of Clemson University was toxic at ca. 1,000 mg kg^{-1} in the mouse bioassay while a sample collected at Lake Worth, Ga., was found to be nontoxic. Benthic *Lyngbya* isolates from the Murray-Darling Basin in Australia were found to be nontoxic (4). Humpage et al. (15) have suggested that some of the unidentified cyanobacterial neurotoxins that were detected in studies might also have been PSPs.

The observation of signs of poisoning in the mouse bioassay led to the use of chromatographic techniques for separating the toxin(s). The signs observed in the mouse bioassay indicated the presence of either antx-A, PSPs, or an unknown cyanobacterial neurotoxin. Tests of crude *L. wollei* extracts for chromatographic characteristics of antx-A (13) were negative. Assays of the PSPs were then attempted. Although the AOAC method for determination of PSPs is a mouse bioassay (3, 27), HPLC has proven to be the method of choice for both the qualitative and quantitative determination of the STXs. The development of the HPLC methodology was initially impeded by the lack of a chromophore. Since the net charges of the various PSPs range from −1 to +2 at ca. pH 7.0, conditions have not been found in which all of the PSPs can be separated in a single HPLC run. These toxins are usually separated on the basis of the number of sulfate groups on the molecule. The *N*-sulfocarbamoyl-11-hydroxysulfate compounds, or C toxins,

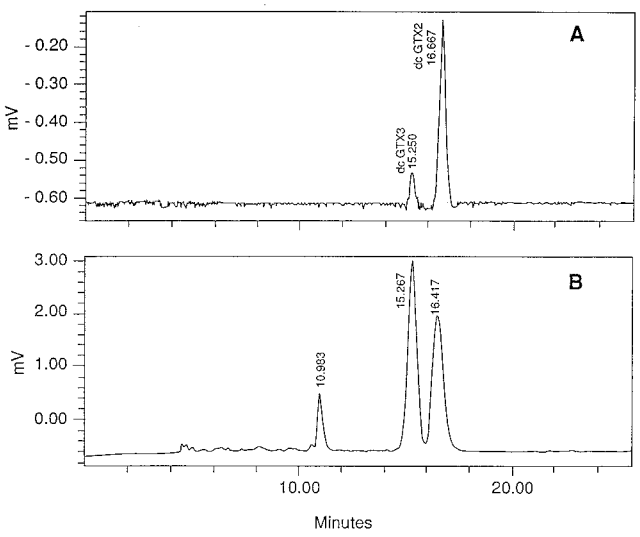
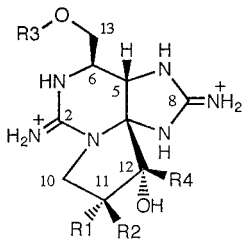


FIG. 5. HPLC chromatograms of decarbamoyl GTX2 (dc GTX2) and dc GTX3 (A) and the toxic fraction eluting from the Supelco C_{18} LC-ABZ column at 7 to 9 min (B). The mobile phase consisted of 10 mM ammonium phosphate–2 mM heptane sulfonate, pH 7.1.



	R1	R2	R3	R4
STX	H	H	$CONH_2$	OH
GTX3	H	OSO_3^-	$CONH_2$	OH
dcSTX	H	H	H	OH
dcGTX2	OSO_3^-	H	H	OH
dcGTX3	H	OSO_3^-	H	OH

FIG. 6. Structures of decarbamoyl GTX2 (dcGTX2) and dcGTX3, the two PSPs found, on the basis of HPLC coelution, in *L. wollei*, plus dcSTX, which also may be present. STX and GTX3 are included for reference purposes.

are separated as one group, the monosulfate toxins (either *N*-sulfocarbamoyl or 11-hydroxysulfate), or gonyautoxins, are separated as another group, and the non-sulfate-containing STXs are separated as the third group. A fluorometric procedure that involves postcolumn chemical oxidation of the toxins (6) is the basis of the HPLC techniques employed to analyze most PSPs. The oxidation reaction is fairly specific for the PSPs, but not all of the toxins produce the same amount of fluorescence. Ion interaction HPLC on porous polymer columns (29) and silica-based reversed-phase columns (17, 25) have been employed to separate and quantify the PSPs, with the utilization of ion-pairing reagents in the mobile phases. With these polymer columns, the C toxins are separated with one mobile phase and the STXs and GTXs are separated with another mobile phase. Most HPLC methods utilizing reversed-phase columns employ three isocratic mobile phases to separate the STXs, the GTXs, and the C toxins (25). It was for these reasons that multiple mobile phases were used in the work presented here.

The HPLC studies presented here show that the major PSP-like compounds present in *L. wollei* share chromatographic retention characteristics with the decarbamoyl toxins (Fig. 6). The decarbamoyl toxins had previously been found in littleneck clams (29). It was suggested that the enzymes responsible for the decarbamoyl reactions might be unique to this particular species of shellfish, but subsequent studies have found the decarbamoyl toxins to be in other shellfish and in the cyanobacterium *Anabaena circinalis* (15). The toxin profile of *L. wollei* does not resemble the profile of either *Aphanizomenon flos-aquae* or *Anabaena circinalis*. The major PSP toxins found in *Aphanizomenon flos-aquae* are neoSTX and STX (20), while the major toxins in *Anabaena circinalis* are the STX, GTXs, C1 and C2 (4, 15). The toxins observed in *L. wollei* and *Anabaena circinalis* are similar in that neither species produces STXs that are hydroxylated at the N_1 position. There is some evidence that freshwater bivalves also accumulate PSPs (26). Therefore, in those areas where bivalves occur and occurrences of *L. wollei* are also common, it would be useful to know if bivalves accumulate the *L. wollei* PSPs. It will also be important to determine if these *L. wollei* PSPs accumulate or adversely affect other organisms in the reservoir's ecosystem, although at present there is no indication that they do.

Some of the oxidation-dependent fluorescent HPLC peaks in the samples have undergone further characterization. They were analyzed by electrospray ionization mass spectrometry and nuclear magnetic resonance spectroscopy to identify these potentially unknown PSPs (24a). In addition, toxin profiles of the cultured *L. wollei* are being obtained for future publication (30a).

To compliment these chromatographic studies, some more specific biochemical tests for PSPs, involving saxiphilin (a PSP-binding protein) and interaction of crude *Lyngbya* extracts in a [^{3}H]STX binding assays, were done. In addition, comparison of the binding properties (K_D values) and binding competition of the *L. wollei* extracts with PSPs provided evidence that excluded the presence of certain PSPs, e.g., STX and neoSTX, in *L. wollei*. A comparison of Fig. 1A and B showed that pure STX was not present because of its lower affinity for the brain Na channel. neoSTX could not have been present, since in previous studies (19) this STX derivative exhibited approximately 200-fold-lower affinity for the frog saxiphilin than for the garter snake saxiphilin. Conversely, GTX5 (also known as *N*-sulfocarbamoyl STX) had a ~130-fold-lower affinity for the snake saxiphilin than for the frog saxiphilin, which also ruled out its presence. This type of evidence is useful for comparison with the HPLC retention data, which also supported the absence of STX, neoSTX, and GTX5.

The rationale for performing the screening assays for the presence of other cyanobacterial biotoxins in *L. wollei* was based on the fact that cyanobacteria are known for their simultaneous production of both hepatotoxins and neurotoxins (1, 14). Moreover, the presence of peptide hepatotoxins could be masked by the shorter response time of the neurotoxins in the mouse bioassay. The peptide hepatotoxins could be present at levels which do not induce the typical acute symptoms of poisoning in the mouse bioassay but could be detected by the ELISA and the protein phosphatase assay. In addition, the presence of other compounds with tumor-promoting activity (e.g., okadaic acid) would not be detected by the ELISA but would be detected by the protein phosphatase assay (24, 31). No indications of hepatotoxins were found, however.

ACKNOWLEDGMENTS

This study was supported in part by a cooperative agreement between Wright State University and the Tennessee Valley Authority (TV-86899V).

We thank Leon Bates, Wayne Poppy, and Doug Murphy of the TVA for assistance and logistics in collecting samples. We especially thank Lawrence A. Dyck, Clemson University, for field support and helpful discussions regarding *Lyngbya* ecology on Guntersville Reservoir. PSP standards were kindly provided by Sherwood Hall, Food and Drug Administration, Washington, D.C., and Y. Oshima, Tohoku University, Sendai, Japan. Gregory Doucette, National Marine Fisheries Service, Charleston, S.C., provided valuable advice on the fluorometric PSP detection procedures.

REFERENCES

1. **Al-Layl, K. J., G. K. Poon, and G. A. Codd.** 1988. Isolation and purification of peptide and alkaloid toxins from *Anabaena flos-aquae* using high performance thin-layer chromatography. J. Microbiol. Methods **1:**251–258.
2. **An, J., and W. W. Carmichael.** 1994. Use of a colorimetric protein phosphatase inhibition assay and enzyme linked immunosorbent assay for the study of microcystins and nodularins. Toxicon **32:**1495–1507.
3. **Association of Official Analytical Chemists.** 1990. Method 959-08, p. 881–882. *In* Official methods of analysis, 15th ed., vol. 2. Association of Official Analytical Chemists, Arlington, Va.
4. **Baker, P. D., and A. R. Humpage.** 1994. Toxicity associated with commonly occurring cyanobacteria in surface waters of the Murray-Darling Basin. Aust. J. Mar. Freshwater Res. **45:**773–786.
5. **Beer, S., W. Spencer, and G. Bowes.** 1986. Photosynthesis and growth of the filamentous blue-green alga *Lyngbya wollei* in relation to its environment. J. Aquat. Plant Manag. **4:**61–65.

5a. **Bell, P., and E. Moczydlowski.** Unpublished data.

6. **Buckley, L. J., Y. Oshima, and Y. Shimizu.** 1978. Construction of a paralytic

shellfish toxin analyzer and its application. Anal. Biochem. **85:**157–164.
7. **Cardellina, J. H., II, F. J. Marner, and R. E. Moore.** 1979. Seaweed dermatitis: structure of lyngbya toxin A. Science **204:**193–195.
8. **Carmichael, W. W.** 1992. Cyanobacteria secondary metabolites—the cyanotoxins. J. Appl. Bacteriol. **72:**445–459.
9. **Carmichael, W. W., and W. R. Evans.** 1996. Detection, identification and production of paralytic shellfish poisons (PSP) by the mat forming cyanobacterium *Lyngbya wollei* Farlow ex Gomont in Guntersville Reservoir. Final report, Tennessee Valley Authority Joint Agency Guntersville Project. Tennessee Valley Authority, Vicksburg, Miss.
10. **Chu, F. S., and X. Huang.** 1992. Production and characterization of antibodies against neosaxitoxin. J. AOAC Int. **75:**341–345.
11. **Guo, X., A. Uehara, A. Ravindran, S. Bryant, S. Hall, and E. Moczydlowski.** 1987. Kinetic basis for insensitivity to tetrodotoxin and saxitoxin in sodium channels of canine heart and denervated rat skeletal muscle. Biochemistry **26:**7546–7556.
12. **Harada, K.-I.** 1996. Chemistry and detection of microcystins, p. 103–148. *In* M. F. Watanabe, K.-I. Harada, W. W. Carmichael, and H. Fujiki (ed.), Toxic *Microcystis*. CRC Press, Boca Raton, Fla.
13. **Harada, K.-I., I. Nagai, Y. Kimura, M. Suzuki, H. Park, M. F. Watanabe, R. Luukkainenn, K. Sivonen, and W. W. Carmichael.** 1993. Liquid chromatography/mass spectrometric detection of anatoxin-a, a neurotoxin from cyanobacteria. Tetrahedron **49:**9261–9260.
14. **Harada, K.-I., K. Ogawa, Y. Kimura, H. Murata, M. Suzuki, P. M. Thorn, W. R. Evans, and W. W. Carmichael.** 1991. Microcystins from *Anabaena flos-aquae* NRC-525-17. Chem. Res. Toxicol. **3:**473–481.
15. **Humpage, A. R., J. Rositano, A. Bretag, R. Brown, P. Baker, B. C. Nicholson, and D. A. Steffensen.** 1994. Paralytic shellfish poisons from Australian cyanobacterial blooms. Aust. J. Mar. Freshwater Res. **45:**761–771.
16. **Koehn, F. E., R. E. Longley, and J. R. Reed.** 1992. Microcolins A and B, new immunosuppressive peptides from the blue-green alga *Lyngbya majuscula*. J. Nat. Prod. **55:**613–619.
17. **Lawrence, J. F., and C. Ménard.** 1991. Liquid chromatographic determination of paralytic shellfish poisons in shellfish after prechromatographic oxidation. J. Assoc. Off. Anal. Chem. **74:**1006–1012.
18. **Li, Y., and E. Moczydlowski.** 1991. Purification and partial sequencing of saxiphilin, a saxitoxin-binding protein from the bullfrog, reveals homology to transferrin. J. Biol. Chem. **266:**15481–15487.
19. **Mahar, J., G. L. Lukacs, Y. Li, S. Hall, and E. Moczydlowski.** 1991. Pharmacological and biochemical properties of saxiphilin, a soluble saxitoxin-binding protein from the bullfrog (*Rana catesbeiana*). Toxicon **29:**53–71.
20. **Mahmood, N. A., and W. W. Carmichael.** 1986. Paralytic shellfish poisons produced by the freshwater cyanobacterium *Aphanizomenon flos-aquae* NH-5. Toxicon **24:**175–186.
21. **Mahmood, N. A., and W. W. Carmichael.** 1987. Anatoxin-a(s), an anticholinesterase from the cyanobacterium *Anabaena flos-aquae* NRC 525-17. Toxicon **25:**1221–1227.
22. **Matsunaga, S., R. E. Moore, W. P. Niemczura, and W. W. Carmichael.** 1989. Anatoxin-a(s), a potent anticholinesterase from *Anabaena flos-aquae*. J. Am. Chem. Soc. **111:**8021–8023.
23. **Moczydlowski, E., B. M. Olivera, W. R. Gray, and G. R. Strichartz.** 1986. Discrimination of muscle and neuronal Na-channel subtypes by binding competition between [^{3}H]saxitoxin and μ-conotoxins. Proc. Natl. Acad. Sci. USA **83:**5321–5325.
24. **Nishiwaki-Matsushima, R., T. Ohta, S. Nishiwaki, M. Sugamura, K. Kohyama, T. Ishiwaki, W. W. Carmichael, and H. Fujiki.** 1992. Liver cancer promoted by the cyanobacterial peptide toxin microcystin-LR. J. Cancer Res. Clin. Oncol. **118:**420–424.

24a. **Onodera, H., M. Satake, Y. Oshima, T. Yasumoto, and W. W. Carmichael.** Submitted for publication.

25. **Oshima, Y., H. Itakura, K.-C. Lee, T. Yosumoto, S. Blackburn, and G. Hallegraeff.** 1993. Toxin production by the dinoflagellate *Gymnodium catenatum*, p. 907–912. *In* T. J. Smayda and Y. Shimizu (ed.), Toxic phytoplankton blooms in the sea. Elsevier Science Publishers, B. V., Amsterdam, The Netherlands.
26. **Park, D. L., W. N. Adams, S. L. Graham, and R. C. Jackson.** 1986. Variability of mouse bioassay for determination of paralytic shellfish poisoning toxins. J. Assoc. Off. Anal. Chem. **69:**547–550.
27. **Sommer, H. W. F. Whedon, C. A. Kofoid, and R. Stohler.** 1937. Paralytic shellfish poisoning. Arch. Pathol. **24:**537–559.
28. **Speziale, B. J., and L. A. Dyck.** 1992. Lyngbya infestations: comparative taxonomy of *Lyngbya wollei* comb. nov. (Cyanobacteria). J. Phycol. **28:**613–706.
29. **Sullivan, J. J.** 1990. High-performance liquid chromatographic method applied to paralytic shellfish poisoning research, p. 66–77. *In* S. Hall and G. Strichartz (ed.), ACS Symposium Series vol. 418. Marine toxins. American Chemical Society, Washington, D.C.
30. **Tokai, A., and G. Mieskes.** 1991. Inhibitory effect of okadaic acid on the p-nitrophenyl phosphate phosphatase activity of protein phosphatases. Biochem. J. **275:**233–239.

30a. **Yin, Q. Q., W. W. Carmichael, and W. R. Evans.** in press.

31. **Yoshizawa, S., R. Matsushi, M. F. Watanabe, K.-I. Harada, A. Ichiwara, W. W. Carmichael, and H. Fujiki.** 1990. Inhibition of protein phosphatases by microcystin and nodularin associated with hepatotoxicity. J. Cancer Res. **116:**609–614.